ORIGINAL ARTICLE

Dose Optimization of H56:IC31 Vaccine for Tuberculosis-Endemic Populations

A Double-Blind, Placebo-controlled, Dose-Selection Trial

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Abstract

Rationale: Global tuberculosis (TB) control requires effective vaccines in TB-endemic countries, where most adults are infected with *Mycobacterium tuberculosis* (*M.tb*).

Objectives: We sought to define optimal dose and schedule of H56:IC31, an experimental TB vaccine comprising Ag85B, ESAT-6, and Rv2660c, for *M.tb*-infected and *M.tb*-uninfected adults.

Methods: We enrolled 98 healthy, HIV-uninfected, bacillus Calmette-Guérin–vaccinated, South African adults. *M.tb* infection was defined by QuantiFERON-TB (QFT) assay. QFT-negative participants received two vaccinations of different concentrations of H56 in 500 nmol of IC31 to enable dose selection for further vaccine development. Subsequently, QFT-positive and QFT-negative participants were randomized to receive two or three vaccinations to compare potential schedules. Participants were followed for safety and immunogenicity for 292 days.

Measurements and Main Results: H56:IC31 showed acceptable reactogenicity profiles irrespective of dose, number of vaccinations, or *M.tb* infection. No vaccine-related severe or serious adverse events were observed. The three H56 concentrations tested induced equivalent frequencies and functional profiles of antigen-specific CD4 T cells. ESAT-6 was only immunogenic in QFT-negative participants who received three vaccinations.

Conclusions: Two or three H56:IC31 vaccinations at the lowest dose induced durable antigen-specific CD4 T-cell responses with acceptable safety and tolerability profiles in *M.tb*-infected and *M.tb*-uninfected adults. Additional studies should validate applicability of vaccine doses and regimens to both QFT-positive and QFT-negative individuals.

Clinical trial registered with www.clinicaltrials.gov (NCT01865487).

Keywords: tuberculosis; H56; subunit vaccine; QuantiFERON-TB (QFT); clinical trial

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A complete list of H56-035 Trial Group members may be found before the beginning of the References.

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At a Glance Commentary

Scientific Knowledge on the Subject: Vaccination against tuberculosis (TB) must protect *Mycobacterium tuberculosis* (*M.tb*)-infected adults in high-burden countries if it is to interrupt the cycle of transmission and achieve World Health Organization goals for TB control. It follows that in countries with high force of infection like South Africa, a TB vaccine will be required to boost antimycobacterial responses in *M.tb*–infected and –uninfected individuals alike.

What This Study Adds to the Field: The study reports a dose-selection clinical trial to determine the optimal vaccine dose and vaccination regimen for the subunit vaccine H56, particularly in populations with high levels of exposure to *M.tb*, such as South Africa.

Tuberculosis (TB) is the leading cause of mortality due to a single pathogen globally (1). The only licensed vaccine against TB disease is bacillus Calmette-Guérin (BCG), which protects children against miliary and meningitic TB, but it offers inconsistent protection against pulmonary TB (2, 3). BCG is efficacious in infants but not adults, who are the primary drivers of TB transmission when infected with *Mycobacterium tuberculosis* (*M.tb*) (2, 3). Globally, 1.7 billion people are estimated to be infected with *M.tb*, representing a massive reservoir of individuals at risk of disease and transmission (4). In high-burden countries more than three-fourths of the adult population may be *M.tb*-infected, despite universal infant BCG vaccination (5). Therefore, successful vaccination strategies to control the global TB epidemic would ideally induce a protective immune response in previously BCG-vaccinated adolescents and adults irrespective of *M.tb* infection status (6).

The H56:IC31 vaccine was designed based on a novel multistage vaccine concept targeting different stages of infection (7). It is intended to protect both before and after exposure as a heterologous boost vaccine for BCG-vaccinated individuals in settings endemic for TB (8). H56 is a fusion protein of three mycobacterial antigens: Ag85B, ESAT-6, and Rv2660c (9). Genomic analysis shows that Ag85B is present in *M.tb* and BCG, whereas ESAT-6 and Rv2660c are only present in *M.tb* (7). Ag85B is highly expressed during bacterial replication and a major target for T cells during acute *M.tb* infection in mice. ESAT-6 is highly immunogenic in human and animal models of TB and is expressed at high levels during acute and chronic stages of murine *M.tb* infection (10). Rv2660c is a hypothetical *M.tb* protein with unknown function that may be a good antigenic target during *M.tb* latency because Rv2660c transcripts are highly upregulated upon nutrient starvation (11–14). The H56 fusion protein is formulated in IC31 adjuvant, which consists of NH_2-KLK_5KLK-COOH peptide and the TLR9 agonist ODN1a oligonucleotide (15).

H56:IC31 was immunogenic and, when administered after BCG, reduced the *M.tb* burden and pulmonary pathology in mice (7) and nonhuman primates (16, 17). A first-in-human phase I clinical trial showed that either 15-μg:500-nmol or 50-μg:500-nmol doses of H56:IC31 were safe and immunogenic in a small group of 8 *M.tb*-uninfected and 17 *M.tb*-infected individuals from South Africa (ClinicalTrials.gov NCT01967134) (9). However, additional dose and schedule selection had not been performed for H56:IC31 in *M.tb*-infected and/or *M.tb*-uninfected individuals. The optimal IC31 adjuvant concentration was found to be 500 nmol in a recent dose escalation trial with the related H4:IC31 vaccine (11, 18). In this study, we report the tolerability, safety, and immunogenicity results of a phase I/IIa trial aimed to select H56:IC31 dose and schedule for further development targeting HIV-uninfected adults living in TB-endemic settings. Some of the results of this study have been previously reported in the form of abstracts (19).

Methods

Study Participants

Participants were recruited at the South African Tuberculosis Vaccine Initiative field site near Cape Town, South Africa. This double-blind, placebo-controlled, dose-selection trial enrolled healthy, HIV-uninfected adults, aged 18 to 50 years who had received BCG vaccination at birth. Individuals who had previously received any investigational product, tuberculin skin test, blood, or antibody transfusion within 21 days of study, or had positive HIV, hepatitis B, or hepatitis C serology, signs of acute illness, or history of chronic disease or active TB disease were excluded. Participants were screened using the QuantiFERON-TB (QFT) Gold in-tube test (Qiagen) and stratified as *M.tb*-uninfected or *M.tb*-infected using the manufacturer's positivity cutoff of 0.35 IU/mL.

Vaccinations and Study Groups

H56 fusion protein (5, 15, or 50 μg) was formulated in 500 nmol of IC31 adjuvant, delivered intramuscularly. The study was divided into primary dose selection and secondary schedule selection phases (Table E1 in the online supplement and Figure 1). The primary dose selection phase (group 1) enrolled *M.tb*-uninfected individuals whereas the secondary schedule selection phase (groups 2–4) enrolled *M.tb*-infected and uninfected persons. Participants in group 1 (QFT-negative) received two injections, on Study Days 0 and 56, and were randomized to receive saline placebo ($n = 5$) or 50 μg:500 nmol (group 1a, $n = 15$), 15 μg:500 nmol (group 1b, $n = 15$), or 5 μg:500 nmol (group 1c, $n = 15$) H56:IC31. For the secondary schedule selection phase, participants were stratified by QFT status and randomized to receive three injections of either placebo or 5 μg:500 nmol H56:IC31 on Study Days 0, 56, and 112 (group 2, QFT-negative; group 4, QFT-positive) or two injections of either placebo or 5 μg:500 nmol H56:IC31 on Days 0 and 56 (group 3, QFT-positive) (Figure 1). Vaccinees were observed for 60 minutes after injection. Solicited and unsolicited adverse events (AEs), classified by severity and relatedness to study vaccine, were recorded until Day 28 after injection. Vaccinees were followed up for serious AEs for 292 days.

Ethics Statement

The Medicines Control Council of South Africa and University of Cape Town Human Research Ethics Committee approved protocol and amendments (Ref. 046/2013). Participants provided written informed consent. We adhered to the Declaration of Helsinki and Good Clinical Practice guidelines.

Immunogenicity Analysis

Vaccine immunogenicity was measured using a qualified 12-hour whole-blood

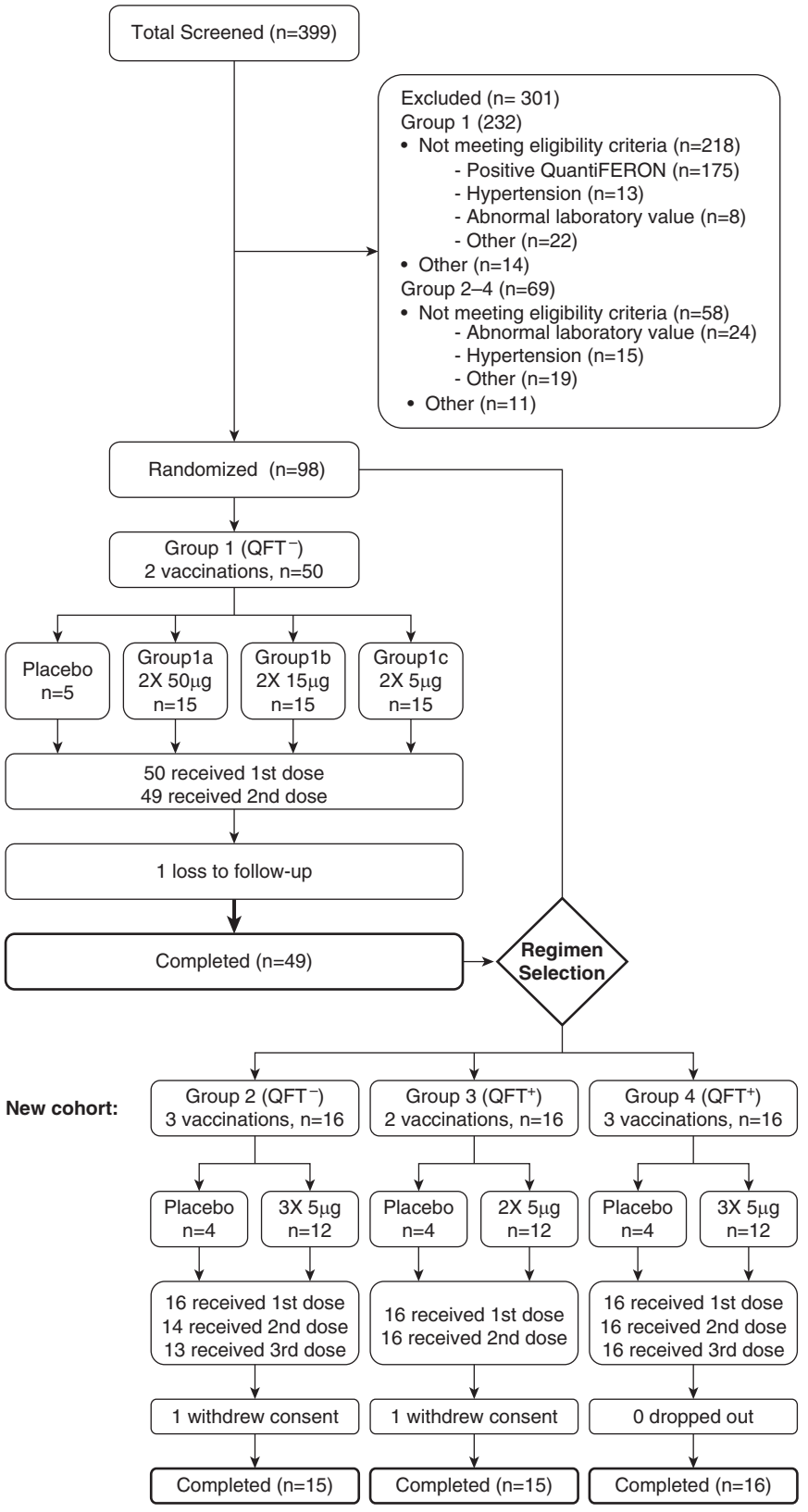

Figure 1. Consolidated Standards of Reporting Trials diagram of participant recruitment, assessment for eligibility, enrollment into different groups, and completion of follow-up. Two individuals in group 2 did not receive the second vaccination, and three did not receive the third vaccination.

intracellular cytokine staining (WB-ICS) assay (20, 21). Briefly, 1 ml of blood was stimulated with peptide pools spanning Ag85B, ESAT-6, and Rv2660c proteins, PHA (positive control) or left unstimulated (negative control) (the gating strategy is shown in Figure E1. Peripheral blood mononuclear cell ICS (PBMC-ICS) was performed as described (22). IFN-γ enzyme-linked immunospot (ELISPOT) assay was also performed. Details are in the online supplement.

Statistical Analysis

Data were analyzed using an intention-to-treat (13) strategy, thereby including all available time points (23). Antigen-specific cytokine-expressing CD4 T-cell counts were analyzed using COMPASS (Combinatorial Polyfunctionality Analysis of Antigen-Specific T-Cell Subsets) (24, 25). Functional differentiation scores (FDSs) were calculated as ratios of frequencies of antigen-specific IFN-γ^+ over IFN-γ^- Th1 CD4 T cells (26). Details are in the online supplement.

Results

Enrollment and Demographic Criteria

A total of 399 adults were screened, and 98 were enrolled. Reasons for exclusion are described in Figure 1. There were no notable demographic differences between study groups (Table 1).

Safety and Reactogenicity of H56:IC31 by Dose, *M.tb* Infection Status, and Number of Administrations

No vaccine-related severe toxicities or serious AEs or deaths were observed. To select H56:IC31 vaccine dose, we first compared the safety profile of three different doses in group 1 QFT-negative participants. Minor variations in rates of injection site pain and systemic complaints (fatigue, chills) were observed, but were not clinically meaningful (Table 2). The safety profiles of two administrations of the 5:500 H56:IC31 dose were compared between QFT-negative and QFT-positive participants (groups 1c and 3). Minor variations in the rate of injection site pain were observed but were not clinically meaningful (Table 2). Similarly, no clinically meaningful differences in AE rates were observed between QFT-positive and QFT-negative participants receiving three vaccinations at

Table 1. Demographic and Baseline Characteristics of Participants in the Primary Dose-Selection Phase and Secondary Schedule Selection Phase in Which Two or Three Vaccinations Were Compared at the 5-μg:500-nmol Dose Level

Variable	Group 1: QFT-Negative, 5/15/50 μg:500 nmol or Placebo, Two Vaccinations (*n* = *50*)	Group 2: QFT-Negative, 5 μg:500 nmol or Placebo, Three Vaccinations (*n* = *16*)	Group 3: QFT-Positive, 5 μg:500 nmol or Placebo,Two Vaccinations (*n* = *16*)	Group 4: QFT-Positive, 5 μg:500 nmol or Placebo, Three Vaccinations (*n* = *16*)
Median (range) age, yr	23.0 (18–46)	21.5 (18–44)	26 (20–49)	25.5 (19–44)
Sex, *n* (%)				
Female	31 (62.0)	10 (62.5)	4 (25.0)	3.(18.8)
Male	19 (38.0)	6 (37.5)	12 (75)	13 (81.3)
Ethnicity, *n* (%)				
Black	22 (44.0)	5 (31.3)	5 (31.3)	6 (37.5)
Mixed race	28 (56.0)	11 (68.8)	11 (68.8)	10 (62.5)
Median (range) BMI, kg/m^2	24 (16-49)	24 (17-57)	26 (19-45)	25 (19-35)

Definition of abbreviations: BMI = body mass index; QFT = QuantiFERON-TB.
The primary dose-selection phase included *Mycobacterium tuberculosis*–uninfected (groups 1a, 1b, and 1c), active, and placebo recipients combined; the secondary schedule selection phase included *Mycobacterium tuberculosis*–infected and *Mycobacterium tuberculosis*–uninfected (groups 2–4), placebo, and active recipients combined.

the same 5:500 dose level (Table 2, groups 2 and 4). Finally, there were no clinically meaningful differences in safety or reactogenicity profiles in QFT-positive participants who received two or three vaccinations at the same 5:500 H56:IC31 dose (groups 3 and 4; Table 2). Most AEs were mild or moderate in severity, and there did not appear to be an increase in severity of AEs with successive doses of H56:IC31 (Table E3). When participants were stratified solely by QFT status or number of doses, no clinically meaningful differences in related AE rates were associated with QFT status or vaccination schedule (Table E4).

Immunogenicity in QFT-Negative Participants at Different H56:IC31 Doses

To compare immunogenicity of H56:IC31 between different H56 dose levels, we measured frequencies of antigen-specific CD4 and CD8 T cells coexpressing IFN-γ, TNF-α (tumor necrosis factor-α), IL-2, and/or IL-17 by WB-ICS in blood from QFT-negative individuals in group 1, stimulated with Ag85B, ESAT-6, or Rv2660c (Figures 2 and E2). Frequencies of antigen-specific cytokine-positive CD4 T cells appeared to peak at Day 70 for H56:IC31 recipients (Figure 2A). Frequencies of Ag85B-specific cytokine-expressing CD4 T cells were consistently higher in vaccinated participants than in placebo counterparts at all dose levels (Mann-Whitney vaccinated vs. placebo on Day 70: 5 μg, $P = 0.0009$; 15 μg, $P = 0.01$; 50 μg, $P = 0.0005$). However, frequencies of ESAT-6– and Rv2660c-specific CD4 T cells were not uniformly higher than those of placebo controls (Mann-Whitney vaccinated vs. placebo on Day 70 for ESAT-6: 5 μg, $P = 0.08$; 15 μg, $P = 0.052$; 50 μg, $P = 0.052$; and Rv2660c: 5 μg, $P = 0.12$; 15 μg, $P = 0.006$; 50 μg, $P = 0.05$).

To select a vaccine dose for further use, longitudinal antigen-specific CD4 T-cell responses during the 292-day follow-up period were assessed by analysis of response area under the curve (AUC) measured by WB-ICS. No significant differences in antigen-specific CD4 T-cell frequencies were observed between the different vaccine dose levels (Kruskal-Wallis excluding placebo: Ag85B, $P = 0.54$; ESAT-6, $P = 0.97$; and Rv2660c, $P = 0.34$; Figure 2A). Similar results were observed by PBMC-ICS and IFN-γ ELISPOT assays (Figure E3). Antigen-specific CD8 T-cell responses after H56:IC31 vaccination were very low in magnitude and not markedly higher than those observed in placebo recipients (Figure E2A). No differences in CD8 T-cell responses between vaccine dose levels were observed (Kruskal-Wallis excluding placebo: Ag85B, $P = 0.56$; ESAT-6, $P = 0.29$; and Rv2660c, $P = 0.38$).

Frequencies of antigen-specific IL-17–expressing CD4 T-cell responses were extremely low (data not shown). Therefore, subsequent analyses focused on antigen-specific IFN-γ^+, TNF-α^+, and/or IL-2^+ Th1 responses. No differences in median proportions of Th1 cytokine coexpression profiles in antigen-specific CD4 T-cell responses induced by different H56:IC31 dose levels were observed (Figure 2B and Figure E2B). Furthermore, we computed posterior probabilities for detecting antigen-specific CD4 responses above background using COMPASS at different H56:IC31 dose levels (24). COMPASS functionality and polyfunctionality scores for Ag85B- and ESAT-6–specific CD4 T-cell responses increased markedly after first and second H56:IC31 vaccinations (Figures 2C and 2D). Median peak responses to Ag85B were predominantly observed after the second vaccination (on Day 70), whereas ESAT-6 responses generally peaked after the primary vaccination, on Day 14 (Figures 2C and 2D). COMPASS scores for Rv2660c were not different from those observed in placebo recipients (Figures 2C and 2D). No differences in functionality or polyfunctionality scores for Ag85B- and ESAT-6–specific CD4 T-cell responses were detected between the three H56:IC31 concentrations. In general, Ag85B-specific responses were higher and detectable in more vaccinees than ESAT-6–specific responses. Rv2660c-specific responses were low and only detected in some vaccinees (Figures 2B–2D, E3, and E4). Overall, our data suggest that doses of H56 protein ranging from 5 to 50 μg in IC31 induced equivalent quantitative and qualitative antigen-specific CD4 T-cell responses. Because higher doses of H56:IC31 did not provide additional evident T-cell immunogenicity benefit, and no dose-responsive safety or tolerability signal was observed, a dose of 5 μg:500 nmol was selected for vaccination of groups 2–4 in the second phase of the trial.

Table 2. Related Adverse Events by Group, Dose Level, Number of Vaccinations, and *Mycobacterium tuberculosis* Infection Status

MedDRA Preferred Term	Placebo (All Groups Combined) (*n* = 17)	H56:IC31*					
		QFT-Negative				QFT-Positive	
		Group 1a: Two Doses 50 μg:500 nmol (*n* = 15)	Group 1b: Two Doses 15 μg:500 nmol (*n* = 15)	Group 1c: Two Doses 5 μg:500 nmol (*n* = 15)	Group 2: Three Doses 5 μg:500 nmol (*n* = 12)	Group 3: Two Doses 5 μg:500 nmol (*n* = 12)	Group 4: Three Doses 5 μg:500 nmol (*n* = 12)
Subjects with ⩾1 AE	9 (53)	12 (80)	11 (73)	12 (80)	6 (50)	8 (67)	8 (67)
Local solicited AEs							
Injection site erythema	0	0	0	1 (7)	0	1 (8)	1 (8)
Injection site pain	3 (18)	8 (53)	5 (33)	9 (60)	2 (17)	5 (42)	5 (42)
Injection site swelling	0	0	0	1 (7)	0	1 (8)	1 (8)
Systemic solicited AEs							
Arthralgia	1 (6)	2 (13)	1 (7)	1 (7)	0	0	0
Chills	1 (6)	4 (27)	1 (7)	0	0	0	1 (8)
Fatigue	5 (29)	4 (27)	3 (20)	3 (20)	0	2 (17)	1 (8)
Myalgia	2 (12)	2 (13)	5 (33)	2 (13)	1 (8)	2 (17)	1 (8)
Nausea	1 (6)	3 (20)	2 (13)	1 (7)	1 (8)	0	1 (8)
Pyrexia	1 (6)	1 (7)	1 (7)	0	0	1 (8)	0
Unsolicited AEs							
Alanine aminotransferase increased	2 (12)	0	0	1 (7)	1 (8)	0	1 (8)
Aspartate aminotransferase increased	0	0	1 (7)	0	1 (8)	1 (8)	1 (8)
Blood alkaline phosphatase increased	0	2 (13)	0	0	0	0	0
Blood pressure systolic increased	1 (6)	1 (7)	0	0	1 (8)	1 (8)	1 (8)
Hemoglobin decreased	0	0	0	2 (13)	0	1 (8)	0
Headache	1 (6)	2 (13)	1 (7)	2 (13)	1 (8)	1 (8)	1 (8)
Injection site warmth	0	1 (7)	0	1 (7)	0	0	0
Malaise	0	0	0	1 (7)	0	0	1 (8)
Neutrophil count decreased	1 (6)	1 (7)	0	0	0	0	1 (8)
Proteinuria	0	0	0	1 (7)	1 (8)	0	1 (8)
Respiratory rate increased	0	1 (7)	4 (27)	2 (13)	0	0	0
White blood cell count increased	0	0	0	0	0	1 (8)	1 (8)

Definition of abbreviations: AE = adverse event; MedDRA = Medical Dictionary for Regulatory Activities; QFT = QuantiFERON-TB.
Data are shown as *n* (%).
*Includes adverse events reported by one or more participants who received H56:IC31 across all groups combined.

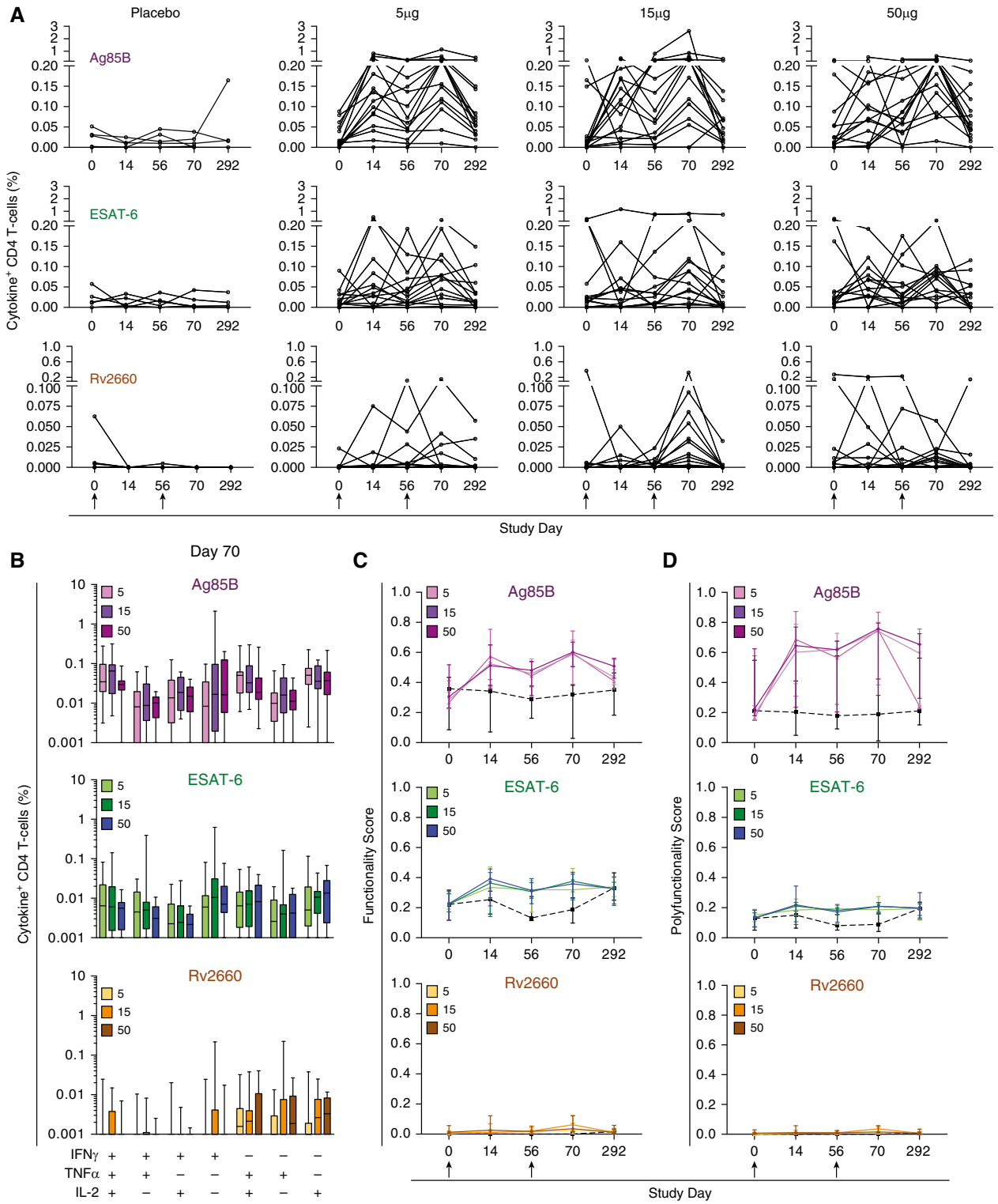

Figure 2. Immunogenicity assessment for H56:IC31 dose selection in *Mycobacterium tuberculosis*–uninfected individuals in group 1. (*A*) Frequencies of antigen-specific CD4 T cells expressing any combination of IFN-γ, TNF-α (tumor necrosis factor-α), IL-2, and/or IL-17 after stimulation with Ag85B, ESAT-6, or Rv2660c measured by whole-blood intracellular cytokine staining assay in participants receiving placebo or different doses of H56:IC31. (*B*) Total frequencies of antigen-specific T-helper type 1 cells coexpressing combinations of IFNγ, TNF-α, and IL-2. IL-17–expressing CD4 T cells were not included in this analysis. Horizontal lines represent medians, boxes the interquartile range, and whiskers the range. Responses are shown for Ag85B, ESAT-6, and Rv2660c on Study Day 70. (*C* and *D*) COMPASS (Combinatorial Polyfunctionality Analysis of Antigen-Specific T-Cell Subsets)-derived functionality (*C*) and polyfunctionality (*D*) scores corresponding to Ag85B-, ESAT-6–, or Rv2660c-specific CD4 T cells. Arrows depict vaccinations with H56:IC31 or placebo.

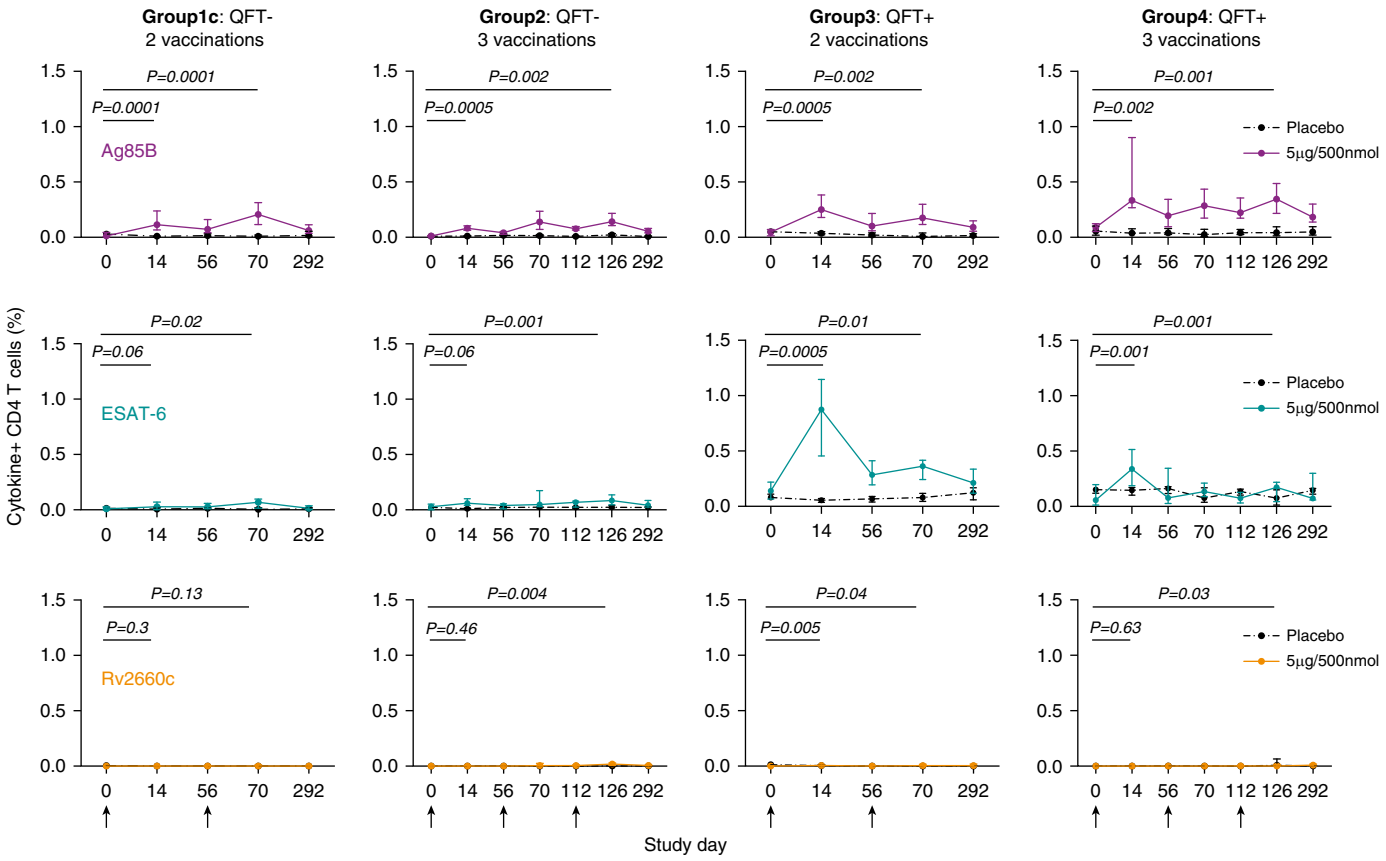

Figure 3. Longitudinal antigen-specific CD4 T-cell responses to two or three administrations of 5-μg:500-nmol H56:IC31 dose in *Mycobacterium tuberculosis*–infected and uninfected individuals. Median frequencies of antigen-specific CD4 T cells expressing any combination of IFN-γ, TNF-α (tumor necrosis factor-α), IL-2, and/or IL-17 during the study follow-up are shown. Error bars denote interquartile ranges. Responses were measured by whole-blood intracellular cytokine staining assay and are shown for Ag85B (top, purple), ESAT-6 (middle, green), and Rv2660c (bottom, orange) and placebo (black dashed lines). Arrows depict vaccinations with H56:IC31 or placebo. *P* values denote comparisons between baseline (Day 0) and Day 14, Day 70 (groups 1c and 3), or Day 126 (groups 2 and 4) responses, calculated using the Wilcoxon signed rank test. The *P* value threshold for significance was adjusted within each group to 0.0083 to account for multiple comparisons (two time points and three antigens were tested per group). QFT = QuantiFERON-TB.

Durability and Functional Profile of H56:IC31-induced CD4 T-Cell Responses

Next, we assessed vaccination schedules comprising either two or three vaccinations of the 5-μg:500-nmol H56:IC31 dose in QFT-positive and QFT-negative individuals. T-cell responses 2 weeks after the first vaccination (Day 14 in all groups) or 2 weeks after the final vaccination (Day 70 in groups 1c and 3, and Day 126 in groups 2 and 4) were compared with prevaccination baseline responses (Figure 3).

Responses to Ag85B were significantly boosted in both QFT-negative (groups 1c and 2) and QFT-positive (groups 3 and 4) vaccinees. Responses to ESAT-6 in QFT-negative participants (groups 1c and 2) increased significantly only after two vaccinations and in particular after three vaccinations (Figure 3). In contrast, in QFT-positive participants, higher frequencies of ESAT-6–specific CD4 T cells were readily detectable after a single vaccination. Rv2660c was poorly immunogenic in both QFT-negative and QFT-positive participants, and only some vaccinees had meaningful CD4 responses after two or three vaccinations (Figure 3).

We determined the durability of H56:IC31-induced antigen-specific CD4 T-cell responses by comparing response frequencies between baseline and the end of the study (Day 292). Ag85B-specific CD4 T-cell responses were maintained at significantly higher frequencies than those at baseline up to Day 292 in QFT-negative and QFT-positive groups (Figure E5). Similar results were obtained by PBMC-ICS and IFN-γ ELISPOT assays (Figures E6 and E7). Day 292 responses to Ag85B were also generally higher in H56:IC31 recipients compared with placebo recipients. Interestingly, frequencies of Ag85B-specific CD4 T-cell responses were higher in QFT-positive than in QFT-negative participants after three, but not two, vaccinations (Figure 4A). Cytokine-expressing ESAT-6–specific CD4 T-cell responses at the end of the study were higher than baseline responses in QFT-positive participants irrespective of the number of vaccinations; however, in QFT-negative participants the end of study ESAT-6 responses were only significantly sustained after three vaccinations (Figure 4).

Vaccination of QFT-negative participants with H56:IC31 resulted in QFT conversion in up to 40% of donors receiving two vaccinations and 64% of donors after three vaccinations (Figure 5A and Table E5). Baseline IFN-γ release was higher in group 1c participants (two doses of 5 μg:500 nmol H56:IC31) who converted to positive QFT after the first vaccination compared with nonconverters (Mann-Whitney; $P = 0.045$, Figure E8), although

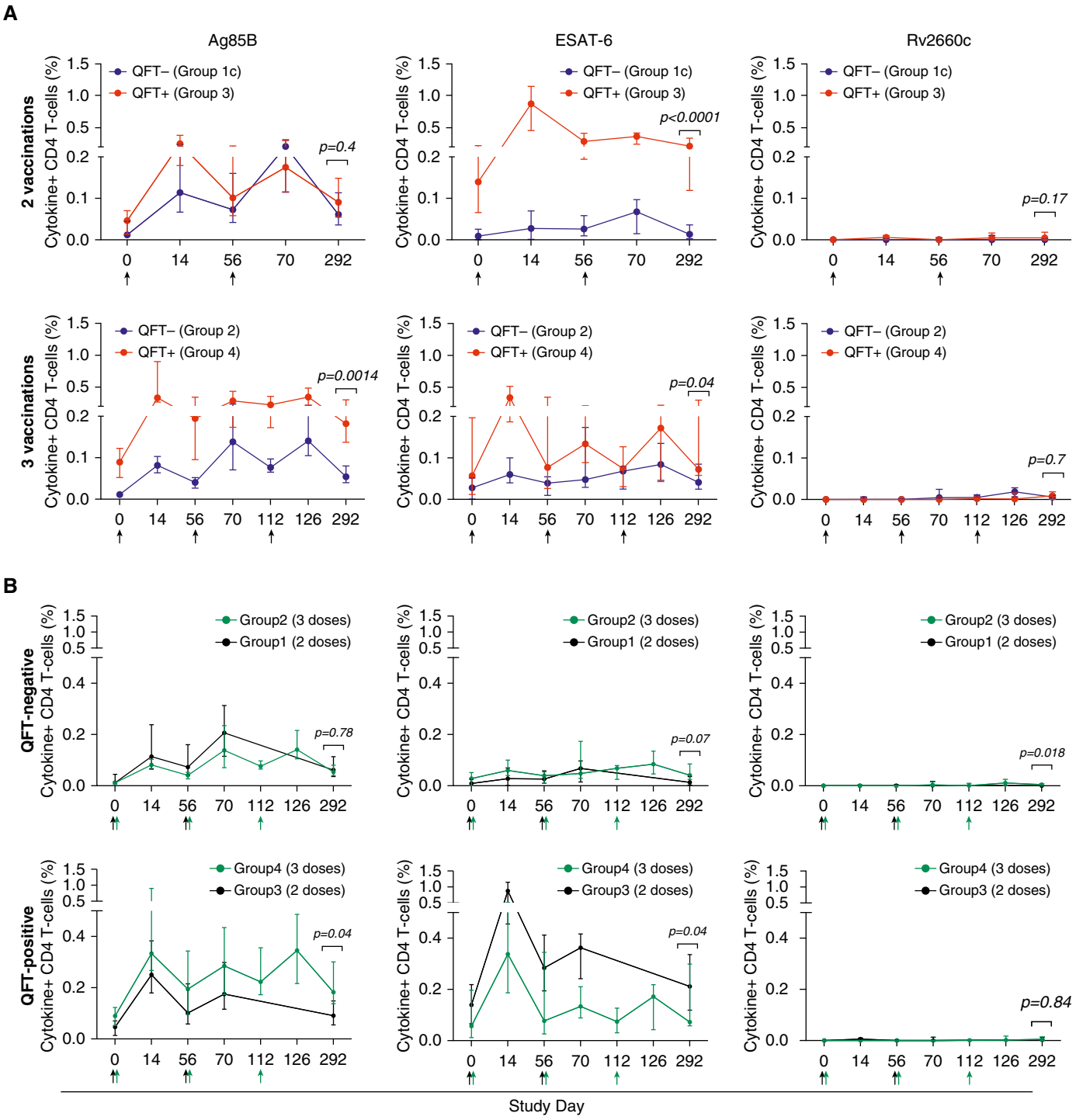

Figure 4. (*A*) Impact of *Mycobacterium tuberculosis* (*M.tb*) infection on immunogenicity of H56:IC31. Median frequencies are shown of Ag85B-, ESAT-6–, or Rv2660c-specific CD4 T cells expressing any combination of IFN-γ, TNF-α (tumor necrosis factor-α), IL-2, and/or IL-17 over time, measured by whole-blood intracellular cytokine staining assay in *M.tb*-uninfected (QuantiFERON-TB [QFT]−, blue lines) or *M.tb*-infected participants (QFT+, red lines). Error bars denote interquartile ranges. Arrows correspond to vaccinations. *P* values represent an intergroup comparison of responses at Day 292, calculated using the Mann-Whitney *U* test. (*B*) Impact of vaccination schedule on immunogenicity of H56:IC31. Median frequencies of Ag85B-, ESAT-6–, or Rv2660c-specific CD4 T cells expressing any combination of IFN-γ, TNF-α, IL-2, and/or IL-17 over time in participants receiving two (black lines) versus three vaccinations (green lines) in either *M.tb*-uninfected (QFT−, upper plots) or *M.tb*-infected participants (QFT+, lower plots). Arrows correspond to vaccinations. *P* values were calculated with the Mann-Whitney *U* test comparing frequencies of antigen-specific CD4 T cells on Study Day 292 between groups receiving two and three vaccinations. The *P* value threshold for significance was adjusted within each group to 0.0167 to account for multiple comparisons (three antigens were tested per group).

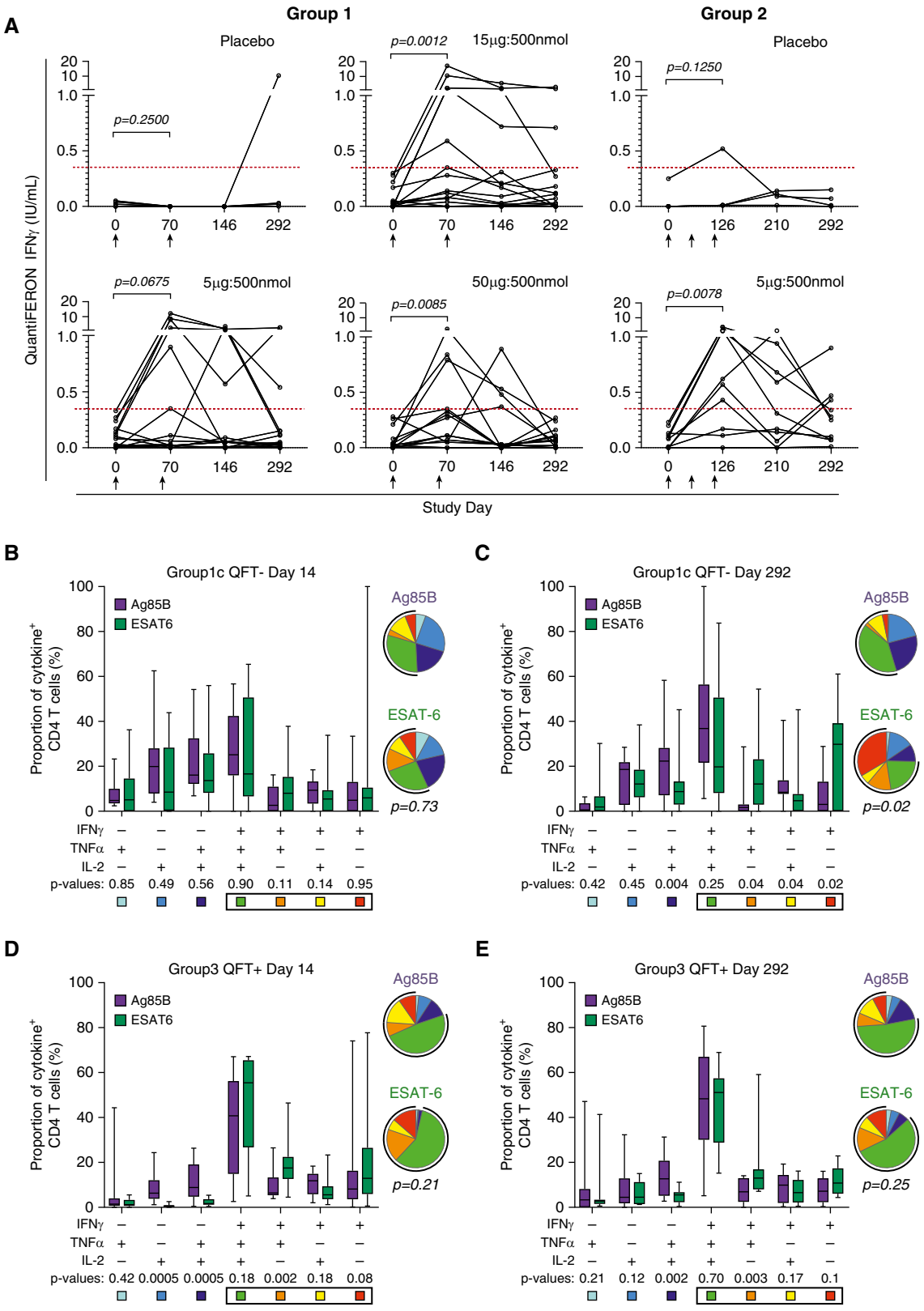

Figure 5. Functional profile of antigen-specific CD4 T cells in *Mycobacterium tuberculosis*–infected and uninfected participants. (*A*) IFN-γ concentrations detected by QuantiFERON-TB (QFT) Gold ELISA in supernatants of blood from participants in QFT-negative groups: groups 1 and 2 were stimulated with tuberculosis antigens (ESAT-6/CFP-10/TB7.7) minus unstimulated background. Groups are stratified by their respective vaccination doses or by placebo.

the sample size was small. Additionally, baseline QFT responses significantly correlated with the magnitude of ESAT-6–specific T-cell responses 2 weeks after the first vaccination (Spearman $r = 0.79$, $P < 0.0001$, Figure E9A). Similarly, QFT responses at the end of the study also correlated with ESAT-6–specific T-cell responses at the same time point (Spearman $r = 0.79$. $P < 0.0001$, Figure E9B).

Two H56:IC31 vaccinations were not sufficient to induce durable cytokine-expressing ESAT-6–specific CD4 T-cell responses (Figure E5). End of the study frequencies of ESAT-6–specific CD4 T cells were also significantly higher than those in placebo recipients, in QFT-negative individuals who received three vaccinations ($P = 0.02$, Figure E5). Durable but very low frequency Rv2660c-specific CD4 T-cell responses were observed in QFT-negative and QFT-positive individuals who received three vaccinations but not in those who received two vaccinations (Figure E5). Collectively, our results suggest that H56:IC31-induced Ag85B-specific CD4 T-cell responses were highly durable, but that ESAT-6 and Rv2660c CD4 T-cell responses were not uniformly maintained up to Day 292.

In addition, we compared the memory phenotype of the durable antigen-specific CD4 T-cell responses in participants who received H56:IC31 at 5 μg:500 nmol at end of the study (Figure E10). Ag85B- and ESAT-6–specific CD4 T cells predominantly expressed a $CCR7^{-}CD45RA^{-}$ effector memory phenotype, whereas a smaller proportion of specific CD4 T cells expressed a $CCR7^{+}CD45RA^{-}$ central memory phenotype. This was true whether participants were QFT-positive or QFT-negative at enrollment (Figure E9). Additionally, H56:IC31-induced Th1 subsets were similar between QFT-positive and QFT-negative participants (Figure E11).

In this trial, the differences between proportions of Ag85B and ESAT-6 Th1 subsets were subtle in the QFT-negative participants who received two vaccinations (Figures 5B and 5C). On Day 292, the proportion of the Ag85B-specific IFN-γ^{-}TNF-α^{+}IL-2^{+} subset was higher than ESAT-6–specific counterparts. In QFT-positive participants who received two vaccinations, both bifunctional IFN-γ^{-}TNF-α^{+}IL-2^{+} and monofunctional IFN-γ^{-}TNF-α^{-}IL-2^{+} subsets were more prominent in Ag85B- than ESAT-6–specific CD4 T cells. Conversely, proportions of IFN-γ^{+}TNF-α^{+}IL-2^{-} ESAT-6–specific Th1 cells were higher than Ag85B-specific counterparts both after first vaccination and the end of the trial (Figures 5D and 5E). Consistently, in QFT-positive participants only, ESAT-6–specific CD4 T cells had a higher FDS, which corresponds to the ratio of IFN-γ^{+} over IFN-γ^{-} antigen-specific Th1 cells (26), than did Ag85B-specific CD4 T cells (Figure E12). Rv2660c-specific Th1 cell frequencies were too low and did not meet the minimum criteria to calculate the associated FDS.

Discussion

H56:IC31 is a subunit candidate TB vaccine, developed as a heterologous boost for QFT-positive and QFT-negative individuals to enhance mycobacteria-specific T-cell responses (7, 16). We report that H56:IC31 was safe, well tolerated, and immunogenic in both QFT-negative and QFT-positive South African adults. In the previous H56:IC31 trial, transient bradycardia was observed among 5 of 32 participants who received 15 μg or 50 μg of H56 in 500 nmol IC31 (9). We did not detect clinically meaningful vaccine-related cardiovascular AEs among participants who received the H56:IC31 vaccine in the present study. We have shown that the lowest H56:IC31 dose of 5 μg:500 nmol provided comparable immunogenicity, safety, and reactogenicity to higher vaccine doses in QFT-negative participants. A three-vaccination schedule induced marginally higher ESAT-6– and Rv2660c-specific CD4 T-cell responses than did two vaccinations in QFT-negative but not QFT-positive participants. Safety and reactogenicity profiles at the selected 5-μg:500-nmol H56:IC31 dose were similar, irrespective of QFT status or vaccination schedule. However, the study sample size was too small to assess rare or infrequent AEs. Importantly, H56:IC31 induced durable antigen-specific Th1 responses irrespective of prior *M.tb* infection.

In a previous trial of H56:IC31 in the same setting, vaccination of QFT-positive participants with 15 μg:500 nmol induced higher frequencies of polyfunctional IFN-γ^{+}TNF-α^{+}IL-2^{+} H56-specific CD4 T cells than with 50 μg:500 nmol, which preferentially induced high frequencies of monofunctional IFNγ^{+} H56-specific CD4 T cells, suggesting skewing to terminally differentiated T cells with limited polyfunctionality (9). Other clinical trials, with H1:IC31 (Ag85B plus ESAT-6) (13) or H4:IC31 (Ag85B plus TB10.4) (27) conducted in the same setting, demonstrated that doses <50 μg:500 nmol induced durable frequencies of antigen-specific Th1 cells and more polyfunctional Th1 cells than did the 50-μg:500-nmol dose. QFT conversion rates in *M.tb*-uninfected H56:IC31 recipients were consistent with observed vaccine-induced ESAT-6–specific responses. Although not statistically different, the observation that three vaccinations were associated with 64% QFT conversion, compared with 40% for two vaccinations, is consistent with an effect driven by ESAT-6–specific vaccine responses. QFT conversion rates were similar to a previous study of H56:IC31 in adults (9), but they were higher than the 15% conversion rates seen after vaccination of adolescents with H1:IC31 (Ag85B plus ESAT-6) in the same setting (13). It remains unknown whether these conversions reflect a (partial) boosting phenomenon of preexisting ESAT-6–specific immunity primed by a well-controlled or self-healed *M.tb* infection, and calls for confirmation in both adolescents and adults in settings with lower endemicity. The inherent issue of vaccine-induced IFN-γ release assay (IGRA) conversion has been addressed by the development of an ESAT-6–free IGRA, which performs with comparable diagnostic accuracy as IGRA and would distinguish

Figure 5. (Continued). *P* values correspond to Wilcoxon signed-rank test between samples taken at prevaccination baseline and 2 weeks after the final vaccination on Day 70 in group 1 and Day 126 in group 2. Arrows correspond to vaccinations. (*B–E*) Proportions of Ag85-specific (purple) or ESAT-6–specific (green) CD4 T cells coexpressing different combinations of the T-helper cell type 1 cytokines IFN-γ, TNF-α (tumor necrosis factor-α), and IL-2 measured by a whole-blood intracellular cytokine staining assay. Analysis was performed on participants receiving two doses of 5 μg:500 nmol of H56:IC31 on Day 14 (*B*) or Day 292 (*C*) in group 1c, and Day 14 (*D*) or Day 292 (*E*) in group 3. Paired analysis between the two antigens was done using the Wilcoxon signed rank test, and *P* values are reported unadjusted. On the right, pie graphs represent median proportions of Ag85B-specific (top) or ESAT-6–specific (bottom) CD4 T cells in the subsets described on the bar graphs on the left, and *P* values apply a permutation *t* test to compare the distribution of the two pies.

M.tb infection from vaccine-induced memory responses (28) (M. Ruhwald, E. Nemes, and J. Kidola, unpublished results). A recent trial of prevention of *M.tb* infection showed promise for the related H4:IC31 subunit vaccine in preventing sustained *M.tb* infection (clinicaltrials.gov identifier NCT02075203) and provides impetus for further clinical development of subunit vaccines like H56:IC31 (29). One placebo recipient in group 1 converted the QFT by the end of the study, likely reflecting newly acquired *M.tb* infection, and a transient conversion was observed in a placebo recipient in group 2, likely reflecting expected fluctuations in the QFT assay (30). IFN-γ expression in antigen-specific Th1 cells was shown to correlate with the degree of T-cell differentiation (31). We recently reported that vaccination with H1:IC31 induced higher proportions of IFN-γ^+ Th1 ESAT-6–specific CD4 T cells in QFT-positive adolescents, relative to Ag85B counterparts (13, 26), similarly to observed ESAT-6–specific CD4 T-cell profiles in this trial. This observation is consistent with the higher expression of ESAT-6 than Ag85B in *M.tb*-infected mice (26, 32). Our data suggest that prior *M.tb* infection increased frequencies of baseline and vaccine-induced antigen-specific CD4 T-cell responses, but had little impact on the quality of the induced response. Therefore, vaccination of QFT-positive adults with higher doses of subunit vaccines containing immunodominant antigens may not be beneficial for maintenance of long-term memory (26). In fact, all participants who received the 50-μg:500-nmol H56:IC31 dose and who converted to positive QFT during the study reverted to negative QFT status by the end of the study, consistent with terminal differentiation of ESAT-6–specific cells. Taken together, these data suggest that a 10-fold lower dose induced qualitatively superior immune responses to those induced by 50-μg:500-nmol doses. Similar dose responses were observed in H56:IC31-vaccinated mice, which showed that the lowest vaccine concentrations (0.1–0.5 μg) were the most immunogenic (33). The lowest H4:IC31 vaccine dose also conferred the most protection against aerosol *M.tb* challenge in mice and guinea pigs (34). These data support dose sparing with protein-adjuvant subunit TB vaccines, and they may support even lower dose ranges than previously tested, as suggested by mathematical modeling of TB vaccine doses (33).

In this study, H56 was administered in the adjuvant IC31, whereas prior studies with small animal models were predominately performed with the cationic liposomal adjuvant CAF01 (7, 35), which generates similar immunogenicity and protective efficacy to H56:IC31 (36). Interestingly, H56:CAF01 vaccination induced high frequencies of ESAT-6–specific lung-homing KLRG1$^-$CXCR3$^+$ CD4 T cells (35). Our data suggest that H56:IC31 induced Th1 cells that predominantly expressed an effector memory phenotype, which lack CCR7 expression associated with lymph node homing, and thus may not be optimal for establishing long-term central memory responses. Nevertheless, we did not test immunological markers associated with lung-homing potential. Of note, a murine study of long-term memory induced by the related H1 (Ag85B and ESAT-6) vaccine candidate showed that functional memory T cells were maintained in draining lymph nodes up to 2 years after vaccination and provided protection against *M.tb* challenge (37). Because it is likely that such long-lived memory T cells reside in lymph nodes in humans also, it is not surprising that such cells were not readily detected in peripheral blood. Long-term follow-up experiments are necessary to explore this further in humans.

This study demonstrates differences in the immunogenic properties of the vaccine antigens. Rv2660c is in H56:IC31, but not in the H1:IC31 vaccine. Immunodominant antigens, such as Ag85B and ESAT-6, are secreted out of the macrophage by *M.tb*, potentially to divert these mycobacterial antigens from presentation (38). Furthermore, prior exposure to *M.tb* (39) or nontuberculous mycobacteria (40, 41) may mask additional immunogenicity conferred by vaccination with antigens to which individuals were previously sensitized. Thus, vaccination with Rv2660c, which is poorly induced by *M.tb* infection (42) and not expressed by BCG, would likely induce *de novo* responses. However, our data suggest that Rv2660c is poorly immunogenic, consistent with both nonhuman primate experiments (16) and the previously reported H56:IC31 trial (9).

Overall, H56:IC31 was safe and immunogenic in QFT-negative and QFT-positive healthy adult volunteers. Because the lowest dose (5 μg) of H56 adjuvanted in 500 nmol of IC31 was immunogenic in *M.tb*-infected and *M.tb*-uninfected adults, this dose was selected for further development. In QFT-positive individuals there was no apparent advantage to three over two vaccinations. The higher ESAT-6–specific CD4 T-cell responses measured by ICS after three vaccinations, as well as the indication of an increased rate of QFT conversion, collectively suggest an advantage of a third immunization in QFT-negative individuals. ■

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